7. (AMENDED) The method of claim 1, wherein the mutagen sensitive gene comprises a variant mutagen sensitive gene.

Please add and consider new claim 57 as follows.

57. (NEW) A method of determining a mutagen comprising:

contacting a test compound with a host cell comprising a DNA sequence encoding a flourescent protein operably linked to an SOS gene;

monitoring a host cell preparation for the flourescent protein; and

determining a mutagen using a Kolmogorov-Smirnov Z Test.

REMARKS

Applicants have received and reviewed the Office Action dated April 25, 2001. By way of response, Applicants have cancelled claims 9, 10 and 28-55 without prejudice, amended claims 1, 6 and 7, and added claim 57. New claim 57 generally corresponds to originally filed claim 1 and includes recitations regarding an SOS gene, a fluorescent protein and analysis using the Kolmogorov-Smirnov Z Test. Claims 1-27 and 56-57 are pending. No new matter is introduced. Applicants submit that the amended and newly presented claims are supported by the specification.

For the reasons given below, Applicants respectfully submit the amended and newly presented claims are in condition for allowance, and notification to that effect is earnestly solicited.

Petition for Extension of Time

It is noted that a three-month petition for extension of time is necessary to provide for timeliness of the response. A request for such an extension is made extending the time for response from July 25, 2001 to October 25, 2001.

Election/Restriction

Applicants acknowledge the Examiner's Restriction Requirement. Applicants have cancelled the unelected claims, claims 28-55, without prejudice.

Nucleotide and/or Amino Acid Sequence Disclosures

Applicants have provided herewith a computer readable form copy of the Sequence Listing and the accompanying statement that the paper and computer readable copies are the same.

Accordingly, it is believed that the application complies with the requirements for patent applications containing nucleotide sequence and/or amino acid sequence disclosures, and notification to that effect is earnestly solicited.

Rejection of Claims Under § 112, Second Paragraph

The Examiner rejected claims 4 and 6 under 35 U.S.C. § 112, second paragraph. In particular, the Examiner objected to the term "variant" in the claims and objected to the change in terminology from "mutagen sensitive" to "mutant sensitive" in claim 6. Applicants respectfully traverse this rejection.

"Mutant sensitive gene"

The Examiner objected to the recitation of "mutant sensitive gene" in claim 6. However, claim 6 does not employ the language cited-- claim 7 does. In response, Applicants have amended the term "mutant sensitive" in claim 7 to recite "mutagen sensitive" to correct the typographic error.

"Variant"

With regard to "variant," Applicants respectfully suggest that the specification as filed supports use of the term. Applicants direct the Examiner's attention to the specification as filed, as discussed below.

a. "Variant SOS gene"

With regard to claim 6, Applicants have amended the claim to recite an "SOS-like gene" as opposed to a "variant SOS gene." This language is directly supported by the specification at least at page 14, lines 10-23, which describes the use of homologues and similar-function genes

in the present invention. By way of clarification, and not to limit the invention, several examples of SOS-like genes are included at least at lines 13-23. Therefore, Applicants respectfully submit that the term "SOS-like" is clearly and adequately defined and supported by the specification.

b. "Variant mutagen sensitive gene"

With regard to claim 7, "variant mutagen sensitive gene" is defined at least on page 15, lines 14-27. For example, at lines 15-16, the specification notes that a variant mutagen sensitive gene is one that retains sensitivity or responsiveness to mutagens. Preferred variants are defined at lines 18-23 as having at least about 80% nucleic acid sequence identity with the naturally occurring or wild-type mutagen sensitive gene. Preferred variants are also described at lines 23-25 as those that hybridize with the naturally-occurring or wild-type mutagen sensitive genes under at least moderately stringent conditions. Finally, at lines 25-27, the specification defines variant mutagen sensitive genes as those including codon and nucleotide substitutions that increase expression or responsiveness in the host cell. The specification also teaches how to make and use variant mutagen sensitive genes at least at pages 15-16. Therefore, Applicants respectfully submit that the term "variant" in connection with mutagen sensitive gene is clearly and adequately defined by the specification.

Conclusion

Accordingly, it is believed that the amended claims fully comply with § 112, second paragraph, and withdrawal of this rejection is respectfully requested.

Rejections Under § 102(a)

The Examiner rejected claims 1-18 and 21-27 under 35 U.S.C. § 102(a) as being anticipated by Justus et al. (Mutagenesis (1999) 14(4): 351-56). Justus et. al. (1999) is not properly considered prior art, as its publication was not prior to the invention disclosed in the application. Applicants have provided herewith a declaration in support of this assertion pursuant to 37 C.F.R. § 1.131. Accordingly, withdrawl of the rejection is respectfully requested.

Rejections Under § 103

The Examiner rejected claims 1-9, 11-14, 16-18, 21-23 and 25-27 under 35 U.S.C. § 103(a) as being obvious over Justus et al. (Mutation Research (1998) 398: 131-41) in view of Chalfie et al. (Science (1994) 263: 802-04). The Examiner rejected claims 1-18, 21-27 and 56 as being obvious over Farr (U.S. Patent 5,589,337) in view of Chalfie et al. (Science (1994) 263: 802-04). The Examiner rejected claims 1-27 and 56 as being obvious over Farr (U.S. Patent 5,589,337) in view of Chalfie et al. (Science (1994) 263: 802-04) in further view of Mitchell et al. (Mutation Research (1986) 159: 139-46). Applicants respectfully traverse these rejections.

Justus et al. (1998) in view of Chalfie et al.

Claim 1 now includes claim 10 and 24, which were not rejected in the Office Action. The rejection is now inapplicable to claim 1 and withdrawl is respectfully requested.

The Examiner asserts in paragraph 11 that "Justus teaches a method of determining a mutagen comprising: . . . b) monitoring a host cell preparation for reporter protein by diluting host cells which are in logarithmic growth and incubating the host cells at 37°C with shaking. . . where the dilution solution may starve the host cell by depleting a nutrient such as dilution into phosphate buffer." By not applying this rejection to claims 10 and 24 relating to host cells in stationary phase, the Examiner has recognized that the method of Justus et al. (1998) requires that the cells be in a metabolically active state at the time of the assay, whereas the present invention has no such requirement. Accordingly, claim 1 has been amended, not to narrow the scope of the claim, but to further clarify that the method disclosed in the present application is not dependent upon the growth phase of the host cell. This reflects a non-obvious advance over the prior art and therefore claim 1, and all claims that depend from claim 1, are now in condition for allowance. ("If an independent claim is nonobvious under 35 U.S.C. § 103, then any claim depending therefrom is nonobvious." MPEP § 2143.03.) Dependent claims 9 and 10 were cancelled as their subject matter is redundant in light of the amendment to claim 1.

Furthermore, one skilled in the art would not be motivated to substitute elements of the method of Justus et al. as there is no suggestion to improve the detection system disclosed therein. The MPEP indicates "a prior art reference must be considered in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention." MPEP § 2141.02, citing *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540. In this case, Justus et al.

(1998) leads one of skill in the art away from the claimed invention by extolling the virtues of its detection system, including "the ease with which it can be used to follow the genetic induction of a well-characterized operon using a simple assay," page 139, column 1, and that the assay is "inexpensive, sensitive and rapid...does not require the preparation of numerous reagents, requires minimal staff training to perform and the sole equipment requirement is for a luminometer," page 139, column 2.

The Office Action further asserts "Justus teaches using analyzing a change in the shape of the data comparing a control cell with the test compound as shown..." However, Applicants' novel conceptualization of "analyzing a change in a shape of a data distribution" in the context of the invention is not contemplated by Justus et al. In fact, the present Application specifically notes that "this type of analysis does not work with the lux reporter," at page 31, lines 17-18. The advantage of the system used in the present invention is that "the present construct can be used to detect subtle mutagens that otherwise might be missed in conventional assays, as well as more potent ones," page 31, lines 18-19. The present invention provides for a statistical means for detecting mutagens "under conditions where there is no significant difference between the arithmetic mean of samples," page 30, lines 29-30. Thus, the present invention is significantly different from, and an improvement over, the methods employed by Justus et. al.

Combining Justus et al. with Chalfie does not cure these deficiencies. The MPEP indicates "the mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination." MPEP § 2143.01 (citing *In re Mills*, 916 F.2d 680). The Examiner asserts that Chalfie suggests the combination. However, the language in Chalfie merely generally touts the benefits of using green fluorescent protein, for example for use in monitoring gene activity and protein distribution within cells. Chalfie does not suggest that its method can function in a mutagen detection system such as that of the present invention. In addition, Chalfie does not provide the limitations missing from Justus et al. regarding the specific data analysis methods of the Application, nor does it provide any indication that green fluorescent protein or other fluorescent proteins can be used specifically to detect mutagens using a construct comprising a mutagen sensitive gene.

In summary, the subject matter as a whole would not be obvious to one having ordinary skill in the art in light of Justus et al. (1998) in view of Chalfie. The method of Justus cannot be

utilized with host cells in stationary phase and does not contemplate a highly sensitive statistical method of analyzing the data. Chalfie et al. does not cure these defects and in addition, does not provide a specific motivation to utilize green fluorescent protein to detect mutagens in combination with a mutagen sensitive gene construct. For at least the foregoing reasons, Applicants respectfully request withdrawl of this rejection.

Farr in view of Chalfie et al.

The Office Action asserts that "it would have been *prima facie* obvious to one having ordinary skill in the art at the time the invention was made to combine the detection method of Farr using a luciferase reporter gene and replace the luciferase reporter gene with the GFP of Chalfie. . ." Applicants note that Farr uses a lacZ fusion protein as a reporter and does not even mention luciferase as asserted by the Office Action.

Again, similar to the argument above, Applicants assert that one skilled in the art would not be motivated to substitute elements of the method of Farr as there is no suggestion to improve the detection system disclosed therein. Although Farr asserts that any assayable product can serve as a reporter, the Farr's examples only disclose use of lacZ. It would not be obvious to one of skill in the art on this basis that fluorescent proteins may be used to assay for stress gene activity. The MPEP § 2143.01 explains "a statement that modifications of the prior art to meet the claimed invention would have been 'well within the ordinary skill of the art at the time the claimed invention was made' because the references relied upon teach all aspects of the claimed invention were individually known in the art is not sufficient to establish a prima facie case of obviousness without some objective reason to combine the teachings of the references," citing *Ex parte Levengood* 28 USPQ2d 1300 (Bd. Pat. App. & Inter. 1993).

Here, the Office Action asserts that Chalfie provides an objective reason to make the combination. However, the language in Chalfie touting the benefits of using green fluorescent protein in monitoring gene activity and protein distribution within cells does not suggest that it will function in the mutagen detection system of Farr.

Further, a rejection based on obviousness requires that there must have been, at the time the invention was made, a reasonable expectation of success in using the references' teachings to make the invention. *See Micro Chem., Inc. v. Great Plains Chem. Co.*, 103 F.3d 1538, 1547 (Fed. Cir. 1997). Nothing in either Farr or Chalfie suggests that practitioners would expect that

GFP could be successfully substituted in Farr's mutagen detection kits. In fact, practitioners have noted problems with the detection of GFP in whole cell suspensions, for example, "One of the problems encountered when measuring whole cell suspensions is that such solutions are extremely turbid. This has the effect of scattering the excitation light in the solution thereby reducing the amount of light reaching the GFP chromophore." www.turnerdesigns.com/t2/doc/appnotes/998_7072.html (copy submitted herewith as Ex. A).

The existence of GFP was known in the art at the time of Farr's disclosure. Yet Farr did not suggest the use of GFP. The Federal Circuit, in rejecting a patentee's argument that its specification was enabled, noted:

> It stands to reason that if the disclosure of a useful conjugate protein and the method for its cleavage were so clearly within the skill of the art, it would have been expressly disclosed in the specification, and in the usual detail.

Genentech, Inc.v. Novo Nordisk, Inc., 108 F.3d 1361, 1367 (Fed. Cir. 1997). By close analogy, if it was obvious to combine GFP with the methods described by Farr it would have been disclosed in the usual detail.

The Office Action further asserts "Farr teaches using analyzing a change in the shape of the data comparing a control cell with the test compound as shown..." However, Applicants' novel conceptualization of "analyzing a change in a shape of a data distribution" in the context of the invention is not contemplated by Farr. The advantage of the system utilized in the present invention is that "the present construct can be used to detect subtle mutagens that otherwise might be missed in conventional assays, as well as more potent ones," page 31, lines 18-19. The present invention provides for a statistical means for detecting mutagens "under conditions where there is no significant difference between the arithmetic mean of samples," page 30, lines 29-30. Thus, the present invention is significantly different from, and an improvement over, the methods employed by Farr.

In summary, the subject matter as a whole would not be obvious to one having ordinary skill in the art in light of Farr in view of Chalfie. The method of Farr does not contemplate a highly sensitive statistical method of analyzing the data. There is no suggestion in either Farr or Chalfie that GFP is suitable for use in the detection of mutagens, nor is there any suggestion that one of skill in the art would have a reasonable expectation of success in making such a

substitution. For the foregoing reasons, Applicants respectfully request withdrawl of this rejection.

Farr in view of Chalfie et al. in further view of Mitchell et al.

For the reasons mentioned, Farr in view of Chalfie does not disclose the present invention. Mitchell does not cure the aforementioned defects. The Office Action asserts that "Mitchell teaches the use of Kolmogorov Smirnov test for the analysis of data regarding the ability of mutagens to effect a reporter system and show a number of Significance levels including $P<.05$." Applicants respectfully assert that this characterization of Mitchell misses the mark. The method of Mitchell does not employ a reporter system at all. Mitchell analyzes morphologic changes in the polychromatic cells of mice fed various agents. There is no suggestion whatever that such statistical analyses employed by Mitchell would be useful in increasing the sensitivity of any type of reporter system. The problem confronted by Mitchell involves *in vivo* mammalian testing. The statistical methods disclosed were used by Mitchell to analyze data from complex living systems. Applicants respectfully suggest Mitchell's conclusion that the K-S test is the most reliable method of analysis for micronucleus assays does not suggest its reliability in other, significantly different assays.

In summary, Farr in view of Chalfie in further view of Mitchell does not disclose the invention of the Application. There is no suggestion in any of the references that they can be successfully combined. For these reasons, Applicants respectfully request withdrawl of the rejection.

Summary

In summary, each of claims 1-27 and 56-57 are in condition for allowance. The Examiner is invited to contact Applicants' undersigned representative at the telephone number listed below, if the Examiner believes that doing so will expedite prosecution of this patent application.

Respectfully submitted,

MERCHANT & GOULD P.C.
P.O. Box 2903
Minneapolis, Minnesota 55402-0903
(612) 332-5300

Date: Oct 25, 2001

Mark T. Skoog
Reg. No. 40,178

Version with Markings to Show Changes Made

1. (AMENDED) A method of determining a mutagen comprising:

contacting a test compound with a host cell comprising a DNA sequence encoding a fluorescent protein operably linked to a mutagen sensitive gene[;], the host cell being in logarithmic or stationary growth phase;

monitoring a host cell preparation for the fluorescent protein; and

determining a mutagen when an amount of the fluorescent protein meets or exceeds a predetermined threshold value, wherein determining further comprises statistically analyzing a difference in the location of a data distribution, a difference in a shape of a data distribution, or a combination thereof.

6. (AMENDED) The method of claim 5, wherein the mutagen sensitive gene comprises an [variant] SOS-like gene.

7. (AMENDED) The method of claim 1, wherein the mutagen sensitive gene comprises a variant [mutant] mutagen sensitive gene.

At pages 12-13, please replace the paragraph beginning at page 12, line 20 with the following:

--The following sequences can provide an SOS gene for the DNA construct of the invention. The nucleotide and corresponding amino acid sequences for an *E. coli umuDC* operon encoding proteins functional in UV mutagenesis and including a promoter for this operon have been reported by Perry et al., Proc. Natl. Acad. Sci. USA 82, 4331-4335 (1985) and given GenBank accession number M13387 (SEQ ID NO: [A]1, SEQ ID NO: 2 and SEQ ID NO: 3) and also by Kitigawa et al. *supra* (GenBank accession number M10107). The nucleotide and corresponding amino acid sequences for a *S. typhimurium* plasmid R46 encoding a *mucAB* gene for mucA and mucB proteins have been reported by Hall et al. and given GenBank accession number X16596 (SEQ ID NO: [D]10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13). See also, Kulaeva O.I. et al. J. Bacteriol. 177(10):2737-2743 (1995). Plasmid R46 is the parent

plasmid for plasmid pKM101. The nucleotide and corresponding amino acid sequences for the *E. coli* plasmid pKM101 encoding at least muc genes have been reported by Perry, K.L. et al. and by Tanooka et al. (Proc. Nat. Acad. Sci. USA 82(13):4331-4335 (1985) and J. Bacteriol. 173(9):2906-2914 (1991), respectively) and given GenBank accession numbers D90147 (SEQ ID NO: [C]14, SEQ ID NO: 15 and SEQ ID NO: 16), M13388, and M12287. The nucleotide and corresponding amino acid sequences for a *S. typhimurium* plasmid R394 encoding *mucA* and *mucB* genes have been reported by Woodgate et al. and given GenBank accession number AF039836 (SEQ ID NO: [B]20, SEQ ID NO: 21 and SEQ ID NO: 22). The nucleotide and corresponding amino acid sequences for a *S. typhimurium* LT2 *umuDC* operon have been reported by Smith et al. and Thomas et al. (J. Bacteriol. 172:4694-4978 (1990) and J. Bacteriol. 172:4979-4989 (1990), respectively) and given GenBank accession numbers M57431 and M35010 (SEQ ID NO: [G]17, SEQ ID NO: 18 and SEQ ID NO: 19). See also, Nohmi T. et al., J. Bacteriol. 173(3):1051-63 (1991). The disclosures of each of the GenBank accessions mentioned in this paragraph and the Perry, K.L. et al. reference are incorporated herein by reference.--

At page 13, please replace the paragraph beginning at line 19 with the following:

--Preferred SOS genes include the *umuC* gene and the *umuD* gene with the corresponding control sequence, preferably of *Escherichia coli*. The nucleotide and corresponding amino acid sequences of these genes are shown in Figure 13 (SEQ ID NO: [A]1, SEQ ID NO: 2 and SEQ ID NO: 3) and have been reported by Perry et al., Proc. Natl. Acad. Sci. USA 82, 4331-4335 (1985) and given GenBank accession number M13387 [(SEQ ID NO: A)]. The *umuC* gene and the *umuD* gene are expressed under control of their natural promoter. One promoter controls the expression of both genes. This promoter is located upstream of the *umuD* and *umuC* coding sequences and, although not limiting to the present invention, is negatively regulated by the LexA protein. After DNA damage, LexA protein can be cleaved and the gene activated. The UmuD and UmuC proteins form a novel polymerase that provides the cell with the capacity to polymerize opposite DNA damage lesions.--

At pages 13-14, please replace the paragraph beginning at page 13, line 30 with the following:

--A fragment of this sequence including nucleotides 1 to 968 of SEQ ID NO: [A]1 can be employed in the present constructs and methods as an SOS gene that responds to mutagens and powers expression of a fluorescent protein. A coding sequence for a heterologous protein, such as a fluorescent protein, can be expressed when inserted in place of all of part of the coding sequence of the *umuC* gene and/or the *umuD* gene, or inserted into and in reading frame with either of these coding sequences. Preferably, a heterologous coding sequence is placed into the *umuC* gene coding sequence at a location such as after nucleotide 968 of SEQ ID NO: [A]1.--

At pages 19-20, please replace the paragraph beginning at page 19, line 22 with the following:

-- Suitable genes and coding sequences for wild type and variant green fluorescent proteins are described in Prasher et al., Gene 111, 229-233 (1992) and (GenBank Accession No. M62653) and in Figure 4a of U.S. Patent No. 5,958,713. The following sequences can provide a green fluorescent protein coding sequence for the DNA construct of the invention. The nucleotide and corresponding amino acid sequences for an *A. victoria* green fluorescent protein have been reported by Prasher et al. *supra* with GenBank accession numbers M62654 (SEQ ID NO: [L]4 and SEQ ID NO: 5) and M62653 and by Inouye et al. (FEBS Lett 351(2-3): 277-280 (1994)) with GenBank accession number L29345. The nucleotide and corresponding amino acid sequences for an *A. victoria* green fluorescent protein mutant 3 have been reported by Cormack et al. (Gene *supra* and Microbiology 143(Part 2):303-11 (1997)) and given GenBank accession number U73901 (SEQ ID NO: [J]23 and SEQ ID NO: 24). The disclosures of each of the GenBank accessions mentioned in this paragraph are incorporated herein by reference.--

At page 20, please replace the paragraph beginning at line 4 with the following:

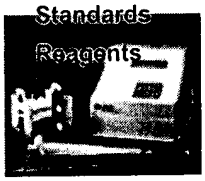
-- A preferred *Aequorea victoria* green fluorescent protein is the variant encoded by a polynucleotide having the sequence shown in Figure 14 (SEQ ID NO: [2]6), or a degenerate sequence encoding the same amino acid sequence. A preferred degenerate sequence employs codons optimized for expression in the host cell. The amino acid sequence of the preferred green fluorescent protein is also illustrated in Figure 14 (SEQ ID NO: [3]7). This sequence has been reported as mut2 by Cormack et al. Gene (1996). A portion of this sequence can be PCR amplified using the method of Matthysse et al (1996) using primers such as *gfpHindIII-F* (5'-

CTCAAGCTTGATTTCTAGATTTAAGAAGG) (SEQ ID NO: [_____]8) and *gfpEcoRi-R* (5'-CTCGAATTCTCATTATTTGTATAGTTCATCCATGCC) (SEQ ID NO: [_____]9) to generate a 740 base pair product.--

At page 24, please replace the paragraph beginning at line 6 with the following:

--Figure 15 illustrates another preferred plasmid, pTJgfp. The plasmid includes a preferred umuDC gene (SEQ ID N[o]O: 1) and a coding sequence for a preferred variant green fluorescent protein (SEQ ID N[o]O: [3]6). It also includes a colE1 replication origin, ori, and a Bla coding sequence for a 9-lactamase selectable marker. The structure and construction of pTJgfp are described in the Examples below and illustrated in Figure 15.--

Application Notes



Learn more about the TD-700 Fluorometer

A TD-700 Laboratory Fluorometer Method for

The Assaying of Green-Fluorescent Protein in Whole Bacteria

Introduction

The green-fluorescent protein (GFP) has become an extremely exciting and useful marker for gene expression. This note describes a method to assay whole living E. coli cells for the presence of GFP using the Turner Designs TD-700 Laboratory Fluorometer. It will arrive at an estimate of mature GFP in viable cells collected from liquid broth.

This is different from quantitating GFP in cell extracts and at various stages of purification. One of the problems encountered when measuring whole cell suspensions is that such solutions are extremely turbid. This has the effect of scattering the excitation light in the solution thereby reducing the amount of light reaching the GFP chromophore. In addition, light scattering may contribute to false emission readings. To overcome these problems, we calibrated the TD-700 against a GFP concentration curve in cell solutions that do not produce GFP, and in solutions prepared and read in a frosted 13mm x 100mm borosilicate cuvette. One of the drawbacks with measuring GFP in whole cells as it is being produced is that its chromophore forms slowly in the presence of molecular oxygen (O2). As a consequence, any direct measurement is probably an estimate of the total GFP present since a portion of the GFP in whole cells has yet to become fluorescent.

2. Materials Required

- TD-700 Laboratory Fluorometer with standard PMT (P/N 7000-009)
- Near UV Mercury vapor lamp (P/N 10-049)
- 13 mm x 100 mm round test tube holder (P/N 7000-981)
- Optical filters providing excitation at 390 nm (P/N 10-134) and emission at 510-700 nm (P/N 10-109R-C)
- 13mm x 100mm borosilicate glass test tubes (P/N 10-031)
- PBS (Phosphate Buffered Saline) 20 mM phosphate (PO3)4 with 150 mM sodium chloride (NaCl), pH 7.4
- 20 µg solution of recombinant Aequorea Green-Fluorescent Protein from ClonTech Laboratories, Inc. [catalogue # 8360-1] Telephone (650) 424-8222
- Frosted Cuvette: The lower 3/4 of a 13mm x 100mm borosilicate test tube is sanded thoroughly on the outside with #60

Aluminum oxide sand paper (dry), followed by a thorough sanding with #320 silicone dioxide sand paper (wet), to achieve a frosted effect.

3. Fluorometer Calibration

3.1 Set-up

Upon receiving and unpacking your TD-700, prepare it for GFP quantitation.

1. Follow the procedure outlined in your operation manual to install the near UV mercury vapor lamp.
2. Install your GFP filters into the filter cylinder and place it into the fluorometer (remember to wipe any fingerprints off of any filter or cuvette after you handle them). The ports for each set of filters are labeled EX for excitation and EM for emission and each pair of ports is labeled A through D. Choosing one pair of ports, carefully insert your filters. The 390 nm filter has a reflective face which should be installed so that it will face out towards the lamp. Each filter is held in place by a circular rubber grommet, or o-ring.
3. At the ends of the filter cylinder are labeled marks corresponding to the pair of filter ports you have chosen. Insert the filter cylinder into the fluorometer while aligning this mark with the silver alignment mark found on the inside rim of the fluorometer's sample chamber.

3.2 Calibration

1. Close the TD-700 lid and turn the unit on. It will count down 600 seconds to warm up.
2. After the instrument warms up, insert the cuvette holder into the sample chamber. Note that the top of the cuvette holder has an arrow shape molded to the top part of the holder. Orient this arrow pointing toward the silver alignment mark on the inside rim of the sample chamber.
3. You will be performing a multi-optional mode calibration (refer to page 21 in your manual if needed). Press [enter] on the keypad. Enter [1] on the keypad to enter SETUP, then press [1] again to enter MODE. Using the ARROW key to choose the mode, select MULTI-OPTIONAL then press [ESC] to return to the setup menu.
4. Enter [3] to enter the units menu, then use the ARROW key to select µg/mL. Press [enter] to return to the setup menu.
5. Enter [2] to enter the calibration procedure menu, and select DIRECT CONCENTRATION using the ARROW key. Press [enter], then [ESC].
6. Enter [2]. Enter the Max Range you want to use (the default range, 999.9, is fine). Enter [1] to select the range given, or [9] to change it. When you select [1], you will be queried for the number of standards you will be using (between 1 and 5). The suggested dilution range for this method is 5 µg/ml to 1 µg/ml (i.e. five standards) in buffer using a frosted cuvette or in a non-GFP expressing cell suspension, in PBS, in an unfrosted cuvette. Enter the number of standards you will be using, then press

[enter] and insert the first sample to be read. Make sure the volume in the tube is over 3 ml, so it is above the window of the cuvette holder.

7. The fluorometer will ask you if the concentration value is correct. If it is, enter [1], if it is not, enter [9] and input the correct value. When you select [1], the machine will prompt you to select the [*] button to store that sample into memory. The cycle will repeat as it asks you for the next highest standard.
8. When you finish calibrating the lowest standard in your series, you will be prompted for your blank, which will be your cell suspension or buffer solution without any GFP. Place that in the TD-700, and press [enter]. The TD-700 will zero itself. When the value at the right of the display becomes stable, enter [0] as requested. It will record your blank and indicate that the calibration is complete.
9. The TD-700 will give you a direct concentration value when you insert your samples and close the chamber door.

4. Quantitating GFP In Intact Bacteria

4.1 Place the sample of *E. coli* suspension to be assayed for GFP into a 13 mm x 100 mm borosilicate test tube (frosted or unfrosted). If your cells were cultured in media possessing residual autofluorescence, such as LB broth, you can remove it by pelleting the cells using centrifugation and resuspending them in PBS. Always make sure the final volume in the tube is over 3 ml (as the meniscus of the sample will affect your readings).

4.2 Insert into TD-700 chamber and close the chamber door.

4.3 Press [*] to begin reading. Record reading when display reads END.

4.4. Example Quantitation

1. You have a strain of *E. coli* expressing GFP under the control of the lac promoter which was grown in a 55 ml liquid culture under induction overnight. Simultaneously prepare your standard solutions by growing and preparing untransformed E. coli under the same conditions.
2. Pellet 50 ml of the cell suspension and wash several times with 50 ml PBS. Resuspend the cells in 5 ml PBS (which will increase our readings 10-fold).
3. Perform the above mentioned calibration and quantitation procedure (sections 3 and 4) to estimate the concentration of GFP in your transformed cells.
4. Comparison of typical GFP concentration estimates in this example for frosted and unfrosted cuvettes, after considering your 10-fold increase in readings:

   Tube Type = unfrosted, Estimated Concentration = 0.89 µg/ml
   Tube Type = frosted, Estimated Concentration = 0.84 µg/ml

   These numbers are very similar and reflect an estimation of the concentration of mature GFP present in these cells. They also

indicate that using a frosted cuvette instead of suspended cells to calibrate the fluorometer might be a workable alternative when you need to quickly estimate the amount of GFP that is present in your cells.

---

5. References

1. A suggested method for the quantitation of Green-Fluorescent Protein. Turner Designs Application note. 1996.
2. Davis, D. F., W. W. Ward and M. W. Cutler. 1994. Post-translational chromophore formation in recombinant GFP from E. coli requires oxygen. In: Bioluminescence and Chemiluminescence, Fundamentals and Applied Aspects. A. K.Campbell, L. J. Kricka, and P. E. Stanley (eds.) John Wiley & Sons, pp. 596-599.

---

6. About the Authors

This GFP application note was written by Daniel G. Gonzalez M.S., who is currently a Biochemistry Ph.D candidate working in the laboratory of William W. Ward at Rutgers University (New Brunswick, N.J.). His interests include the physical characterization of chromophore formation in a variety of Green-Fluorescent proteins from various organisms. He was assisted by John Covalesky, who has recently graduated from Rutgers University with a B.S. in Biochemistry. William W. Ward is one of the pioneers in GFP research and is still very active in the field. He and Daniel currently use a wide assortment of techniques in fluorescence analysis, protein purification and molecular biology to study and characterize Green-Fluorescent Proteins. The Ward lab prepares and coordinates a series of short courses in biotechnology that features Aequorea GFP as a model protein for purification and molecular manipulation. Information on these courses can be obtained by contacting Daniel:

Phone: (908) 932-9071, ext. 219
E-mail: meton@rci.rutgers.edu

Daniel Gonzalez
Rutgers University, Cook College
Biochemistry and Microbiology
76 Lipman Drive
New Brunswick, NJ 08901-8525

Daniel thanks Turner Designs for making this application note possible and for providing the GFP community with a useful analysis tool in their TD-700 fluorometer.